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Un-silencing of native leptin receptors (LepR) in SF-1 neurons does not rescue obese phenotype in otherwise LepR-deficient mice

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ZUSAMMENFASSUNG

Wir überprüfen, ob die Aktivierung der Leptinrezeptoren (LepR) in Neuronen des ventromedialen Kerns des Hypothalamus (VMH) - speziell diejenigen, die den steroidogenen Faktor 1 (SF1) exprimieren - eine Schlüsselrolle bei der Kontrolle der Energiebilanz spielen. Um die Rolle der LepR in der Steuerung der Energiebilanz zu untersuchen, wurde eine Methode entwickelt, in welcher die LepR durch genetische Manipulation ausgeschalten werden. Mäuse (LepR-loxP), denen der LepR deaktiviert wurde, fehlen funktionelle LepR. Eine Kreuzung mit cre-Rekombinase entfernt die Transkriptionsblockierungsequenz und aktiviert spezifische, native Populationen von LepR. Wir haben LepRloxTB x SF1-cre (KO / Tg +) Mäuse verwendet, um LepR speziell im VMH zu deaktivieren und um zu testen, ob die SF1-Neuronen im VMH eine entscheidende Rolle bei der Wirkung von Leptin auf die Energiehomöostase spielen. Leptin-induziertes pSTAT3 war nur im VMH von KO / Tg + -Mäusen und nicht in anderen Kernen des Hypothalamus vorhanden. Die VMH-Leptin-Signalisierung führte in Chow-gefütterten LepR-defizienten Mäusen zu keiner Verbesserung der Fettleibigkeit. Beim Vergleich von LepR-Null-Mäusen mit KO / Tg + -Mäusen, veränderte sich weder die Nahrungsaufnahme oder der Energieverbrauch, noch hat sich die Glukosetoleranz in KO / Tg + verbessert. Das Vorhandensein von funktionellem LepR im VMH erhöhte die Sensitivität gegenüber dem Pankreashormon Amylin nur leicht. Bei einer fettreichen Ernährung (HFD) gab es keine Reduktion der futterinduzierten Fettleibigkeit bei KO / Tg + -Mäusen, jedoch zeigten KO / Tg + -Mäuse nach 6 Wochen auf HFD eine verbesserte Glukosetoleranz verglichen zu den LepR-Null-Mäusen. Diese Daten unterstützen die Idee, dass Leptin das Körpergewicht und die Nahrungsaufnahme reduziert und den Energieaufwand über ein verteiltes Netz von Leptin-reaktionsfähigen Kernen und nicht durch eine einzelne Rezeptorpopulation erhöht. Mäuse, die mit einer HFD gefüttert wurden, haben aufgrund der VMH Leptin Signalisierung eine leicht erhöhte Amylin-Sensitivität und zeigen dadurch eine verbesserte Glucosetoleranz. Die physiologische Relevanz dieser Erkenntnisse bedingt eine weitere Untersuchung. Wir schließen daraus, dass die LepR-Signalisierung im VMH nicht ausreicht, um die beobachtete metabolische Fehlfunktion in LepR-Null-Mäusen zu korrigieren.

SUMMARY

Leptin receptor (LepR) signaling in neurons of the ventromedial nucleus of the hypothalamus (VMH), specifically those expressing steroidogenic factor 1 (SF1), have been proposed to play a key role in controlling energy balance. A recently establsihed experimental tool to explore the role of LepR signaling in controlling energy balance makes use of silencing the LepR trhough genetic manipulations. LepR-silenced (LepRloxTB) mice lack functional LepR, but crossing these mice with cre-recombinase removes the transcription blocking sequence, and un-silences specific, native populations of LepR. We used LepRloxTB x SF1-cre (KO/Tg+) mice to un-silence LepR specifically in the VMH with the aim of testing whether SF1 neurons in the VMH are critical mediators of leptin's effect on energy homeostasis. Leptin-induced pSTAT3 was only present in the VMH of KO/Tg+ mice, whereas this effect was absent in other hypothalamic nuclei. VMH leptin signaling did not ameliorate obesity resulting from LepR-deficiency in chow-fed mice. There was no change in food intake or energy expenditure when comparing complete LepR-null mice to KO/Tg+ mice, nor did KO/Tg+ show improved glucose tolerance. The presence of functional LepR in the VMH mildly enhanced sensitivity to the pancreatic hormone amylin. When maintained on high fat diet (HFD), there was no reduction in diet-induced obesity in KO/Tg+ mice, but KO/Tg+ mice had improved glucose tolerance after 6 weeks on HFD compared to LepR-null mice. These data support the hypothesis that leptin reduces body weight and food intake, and increases energy expenditure, via a distributed network of leptin-responsive nuclei, rather than through a single receptor population. While VMH leptin signaling mildly enhanced amylin sensitivity and improved glucose tolerance when mice were maintained on HFD, the physiological relevance of these effects warrant further investgation. We therefore conclude that LepR signaling in the VMH is not sufficient to correct metabolic dysfunction observed in LepR-null mice.

INTRODUCTION

Leptin, a hormone mainly produced and secreted by the adipocytes of the white adipose tissue, acts in the brain to directly control body weight, food intake, and energy expenditure. Obesity in mice due to a deficiency of leptin (ob/ob) or its receptor (db/db) occurs together with hyperphagia, diabetes and aberrant metabolic homeostasis (19, 28, 32). Leptin exerts its actions by activating the b isoform of the leptin receptor (LepR), which is expressed in various brain regions involved in the control of energy homeostasis. In particular, LepR is highly expressed in hypothalamic structures including the arcuate nucleus (ARH) and the ventromedial nucleus of the hypothalamus (VMH) (8, 9, 15). Studies utilizing various genetic manipulations have focused on how these, and other, central populations of LepR contribute to the control of energy homeostasis.

The ARH is often considered the primary target for leptin in the brain, and the effects of leptin on ARH neurons have been extensively characterized (1, 13). A recently developed mouse model of LepR-deficiency, in which a loxP-flanked transcription blocking (TB) sequence is inserted in the LepR gene ($LepR^{loxTB}$), was used to determine the specific role of leptin signaling on the proopiomelanocortin (POMC) subpopulation of neurons in the ARH. By breeding $LepR^{loxTB}$ mice with mice expressing POMC-cre, native LepR exclusively in POMC-expressing neurons were un-silenced. Reactivation of leptin signaling in this ARH population had no effect in food intake, but it normalized blood glucose and energy expenditure, resulting in a modest correction of obesity associated with global LepR-deficiency. These findings demonstrate that other hypothalamic brain areas contribute to the central mediation of leptin's metabolic effects (1).

Cumulating evidence points to the VMH as a key player in the control of energy metabolism and a target site for leptin (2, 7, 15). Leptin activates VMH neurons as demonstrated by their expression of phosphorylated STAT3 (pSTAT3), a marker of leptin signaling (8). Furthermore, many genes expressed in the VMH are involved in the control of energy homeostasis, notably steroidogenic factor-1 (SF1) (7, 22). SF1 is exclusively expressed in a subpolulation of VMH neurons, and plays a crucial role for the correct development of the VMH and the formation of its efferent and afferent circuits (5, 6, 29). SF1 is also required for the expression of LepR and leptin-responsiveness in the VMH (7, 22). In rodents, a lack of VMH LepR leads to heightened sensitivity to high

fat diet, impaired glucose tolerance and increased levels of insulin and leptin (2, 8), suggesting that these neurons are protective against diet-induced obesity (DIO) and mediate leptin's glucoregulatory effects. The VMH is also the proposed brain site where leptin and the pancreatic hormone amylin interact to produce a synergistic reduction in body weight and food intake (18, 24).

To test exactly what contribution VMH LepR neurons make to leptin's control of energy balance, we bred transgenic $LepR^{loxTB}$ and SF1-cre mice to produce mice with functional LepR exclusively in the SF1 neurons of the VMH. We evaluated the role of leptin signaling specifically in VMH in the control of body weight and composition, food intake, energy expenditure, glucose homeostasis, and amylin sensitivity. Because loss-of-function studies also demonstrate that SF1 neurons expressing LepR might mediate sensitivity to high fat diet (2, 8), we tested if leptin acting in the VMH protects again DIO by challenging the mice with high fat diet.

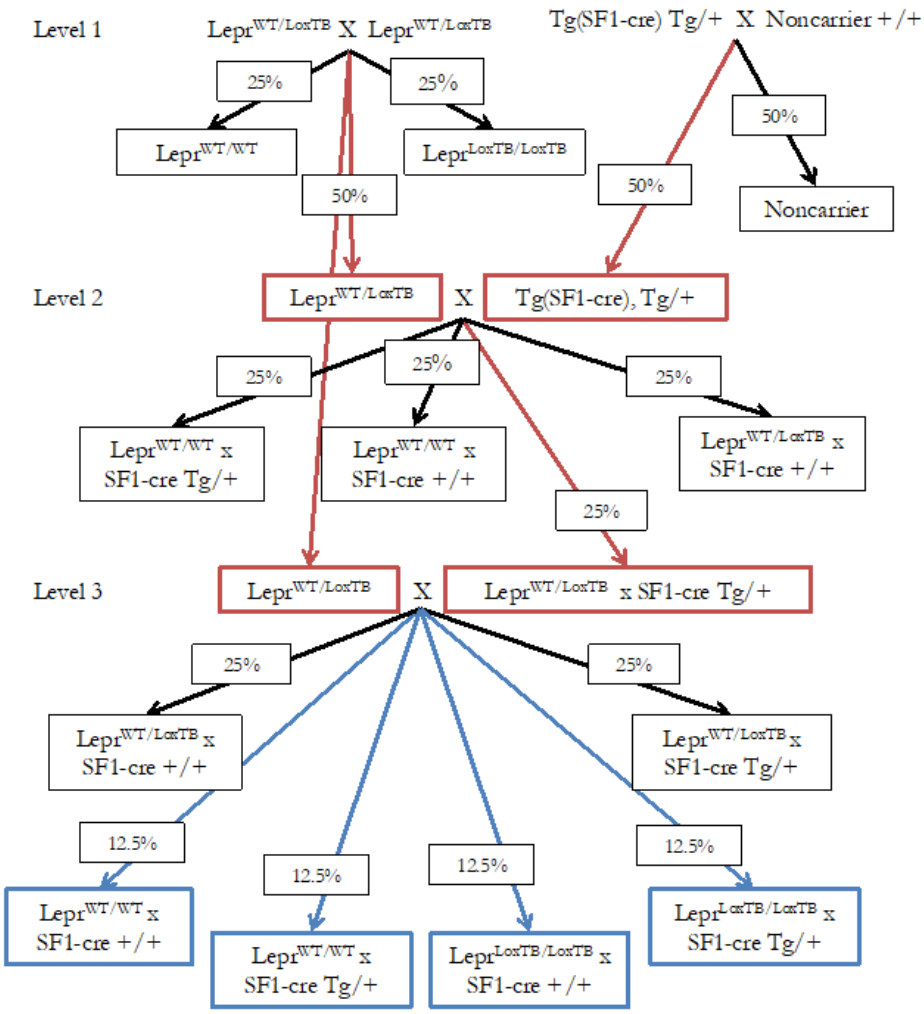
MATERIAL AND METHODS

Generation of mice with un-silenced leptin receptors in SF1 neurons

To generate mice expressing native LepR exclusively in SF1 cells, and otherwise LepR-deficient, the previously validated LepR cre-reactivatable mouse model ($LepR^{loxTB}$; see (1)) was crossed to mice expressing SF1-cre. $LepR^{loxTB}$ breeders were purchased from Jackson Laboratory (Leprtm1Jke; Stock# 018989), and SF1-$cre^{Tg/+}$ mice were generously provided by Joel Elmquist of University of Texas Southwestern Medical Center.

Because homozygous $LepR^{loxTB/loxTB}$ mice are reportedly infertile, a three-level breeding strategy was designed in which the final cross of $LepR^{loxTB/WT}$ to $LepR^{loxTB/WT}$ x SF1-$cre^{Tg/+}$ generated pups representing all four experimental conditions within single litters. The breeding scheme is shown in Figure 1. The four experimental groups generated were: $LepR^{WT/WT}$ x SF1-cre^{WT} (WT/WT), expressing normal LepR and no SF1-cre; $LepR^{WT/WT}$ x SF1-$cre^{Tg/+}$ (WT/Tg+), expressing normal LepR and SF1-cre; $LepR^{loxTB/loxTB}$ x SF1-cre^{WT} (KO/WT), LepR-null, equivalent to db/db mice, and expressing no SF1-cre; and $LepR^{loxTB/loxTB}$ x SF1-$cre^{Tg/+}$ (KO/Tg+), LepR-null, except for the hypothetical reactivation of native LepR only in SF1-expressing cells of the VMH.

Mice were genotyped at the age of 6 or 7 days by double transgenic PCR analysis of toe DNA, which was determined using the following primers (Microsynth AG): TGGCTTTTAAGCTCTGCAGTC ($LepR^{loxTB}$ common), CAAATGTTGCTTGTCTGGTG (SF1 cre Tg-F), GTCAGTCGAGTGCACAGTTT (SF1 cre Tg-R), TGGCTTTTAAGCTCTGCAGTC ($LepR^{loxTB}$ WT) and CCCAAGGCCATACAAGTGTT ($LepR^{loxTB}$ KO).



LepR	WT	WT	KO	KO
SF1-cre	WT	Tg+	WT	Tg+

Figure 1: **Breeding strategy for generating experimental mice.** Three-level breeding plan used to generate mice with LepR un-silenced specifically in SF1 neurons of the VMH, which are otherwise LepR-null, and three control groups. Experimental groups are indicated by blue boxes, and their genotype abbreviation is summarized in the table. Estimated percentage of offspring is according to Mendelian inheritance.

Housing and Environment

Mice were housed in an environment maintained at 21 ± 2°C, under a 12/12 hour light-dark cycles (lights off at 10.00 h). Mice had ad libitum access to standard chow (Kliba Nafag 3430, Kaiseraugst, Switzerland) and water, except when noted below. A subgroup of mice were maintained on high fat diet (HFD; 32% kcal from fat, D12266B from Research Diets) from approximately 5- to 14-weeks of age. The mice were group housed in macrolon cages until 5 to 6 weeks of age, when they were single housed in BioDAQ cages. Each cage was furnished with a cardboard hide including nest-building material. The BioDAQ cages were fitted with a peripheral sensor controller (PSC) to continuously measure individual food intake, and gated food hoppers mounted outside the cage to correct for spillage.

Handling and acclimation

Prior to any testing, mice were given one week to acclimate to new housing. Mice were handled and body weight was monitored at least three times per week throughout the experiment. Mice were adapted to short-term restraint to reduce stress during intraperitoneal (IP) injections.

Peptides

Mouse amylin (Bachem AG; Bubendorf, Switzerland) was reconstituted in sterile NaCl (0.9%) and stored at -20°C until usage. On testing days, amylin was diluted in NaCl to appropriate concentrations and kept on ice until injected. Murine leptin (Peprotech; USA) was stored at -80°C until usage. On testing days, leptin was diluted in PBS (pH 8.4) and kept on ice until injected. IP injection volumes did not exceed 5 ml/kg.

Measurement of baseline food intake and meal patterns

Following 7 days of acclimation in the BioDAQ cages, mice were fasted for 12 hours during the light phase. At dark onset (10.00 h), food was returned and baseline refeeding was measured for the subsequent 22 h. Meal pattern criteria were an inter-meal-interval (IMI) of 600 s and a minimal meal of 0.02g.

Feeding behavior tests

Mice were fasted for 12 hours from the start of the light phase (22.00 h) by closing food hoppers manually. The access to water remained throughout the entire experiment. Two hours before testing, body weight was collected and the BioDAQ system started. The food hoppers were cleaned and refilled with chow. Various doses of amylin (0, 20, 100, 500, 1000 μg/kg) were prepared in 0.9% NaCl and stored on ice. At 9.40 h, the mice were treated with amylin or vehicle (IP) in a randomized crossover procedure where each mouse received each treatment once over 5 test days. At dark onset (10.00 h), the gates of the food hoppers were reopened. Food intake was automatically measured and analyzed for 22-h post-injection.

Intragastric glucose tolerance test (igGTT)

At the ages of 8 and 12 weeks, basal blood glucose was measured and an igGTT was performed. Food was removed from the cages 2 hours before dark onset, at which time, mice were lightly anesthetized under isoflurane, and placed in a supine position. With a cotton-tipped applicator, the tongue was rolled out and the lingual vein was punctured with a 24-G needle. Blood was then collected in a 100-ul EDTA-coated tube, prepared with a protease inhibitor (Sigma P2714; 30 μl of 1:10 diluted stock solution/1 ml blood), and processed for plasma. Approximately 5 μl of whole blood was placed on a glucometer test strip (Glucometer: Breeze2, Bayer, Zurich, Switzerland) to measure baseline blood glucose. Just prior to dark onset, after 2 hours recovery time, the tip of the tail was cut with a scalpel and a second baseline blood glucose was measured. At dark onset, mice were gavaged to administer glucose solution (2 g/kg) directly into the stomach. The time of gavage was recorded and additional blood glucose levels were measured from the tail at 15, 30, 45, 60, 90 and 120 minutes after gavaging. In mice challenged with HFD, an igGTT was performed at 5-weeks of age, prior to access to HFD, and again at 12-weeks of age, after 7 weeks on HFD.

Measurement of baseline energy expenditure and physical activity

Following completion of feeding tests, mice were transferred to the TSE PhenoMaster open circuit indirect calorimetry system for the determination of O_2 consumption and CO_2 production (TSE Systems; Bad Homburg, Germany). Room air was passed through each cage at a flow rate of 0.41 L/min. Every 20 min, cage air was sampled from each individual cage for 1 min 20 sec and analyzed for O_2 and CO_2. From these values, energy

expenditure (EE) and respiratory exchange ratio (RER) were calculated based on equations from Weir (31). To account for differences in body weight and body mass composition, EE data were corrected for individual lean body mass (LBM in g) and fat mass (FM in g) using the following equation: LBM + 0.2FM, as recommended by Even and Nadkarni (10). See also "Measurement of Body Composition."

Perfusion

Following behavioral testing and metabolic phenotyping, mice were fasted 12 hours before they received randomized intraperitoneal amylin (20 μg/kg) or VEH (NaCl 0.9%) injection. Ninety minutes later, the mice were deeply anesthetized with pentobarbital (200 mg/kg IP; injection volume of 5 ml/kg), and mice were perfused transcardially by hydrostatic forces with ice-cold 0.1 M phosphate buffer (PB; pH: 7.2) for 2 minutes, followed by 2 minutes of ice-cold 4% paraformaldehyde (PFA, Sigma-Aldrich) in 0.1M PB to fix the tissue. Brains were removed and were postfixed in 4% PFA overnight at 4°. After 24 h they were transferred to cryoprotectant (20% sucrose solution in 0.1M PB) overnight at 4°C. Brains were then blocked into hindbrain and forebrain, flash frozen in hexane on dry ice, and stored at -20°C until sectioning.

A second cohort of mice was perfused at 8- or 20-weeks of age to validate the expression of functional LepR in the VMH by assessing leptin-induced pSTAT3. Mice were fasted for the last 2 hours of the light phase, and at dark onset all mice were injected with leptin (3 mg/kg, IP). Mice maintained on HFD were treated with leptin (5 mg/kg, IP) at 14-weeks of age. Forty-five minutes later, mice were anesthetized and perfused as above, except that KPBS was used to flush, and 2% PFA was used to fix the tissue. Brains were removed and postfixed in 2% PFA for 1 h, and then cryoprotected and frozen as above.

Brain sectioning

Frozen brains were sectioned through the area postrema (AP) and VMH and ARH at 30 μm on a cryostat (Leica CM3050S, Nussloch, Germany). Coronal brain sections were freeze-thaw mounted on slides and stored in cryoprotective solution (50% 0.1M PB, 30% ethylene glycol, 20% glycerol) at -20°C until staining.

Immunohistochemistry and quantification

Phosphorylated signal transducer and activator of transcription-3 (pSTAT3)

Slide mounted brain sections were washed of cryoprotectant in 0.02 M potassium phosphate buffered saline (KPBS). Sections were then demasked in 1% NaOH (1M) and 1% H_2O_2 (30%) in KPBS for 20 minutes at room temperature, and then incubated in 0.3% glycine in KPBS for 10 minutes at room temperature. Next they were pretreated with 0.03% sodium dodecyl sulfate (SDS), and rinsed in 0.02 M KPBS. Sections were then blocked in 4% normal goat serum and 1% Bovine Serum Albumin (BSA; Sigma-Aldrich) in 0.4% Triton-X 100 (Sigma-Aldrich) in KPBS for 20 minutes at room temperature. Slides were incubated with anti pSTAT3 (1:1000 Santa Cruz Biotechnology, Texas, USA) in 1% NGS, 0.4% triton-X 100 and 1% BSA in KPBS for 48 hours at 4°C. After the incubation with the primary antibody, slides were washed in 0.02 KPBS, then incubated with the secondary antibody Cy3-goat-anti-rabbit (1:1000; Jackson Laboratories, Pennsylvania, USA) in 1% NGS, 0.4% Triton-X 100 and 1% BSA in KPBS for 2 hours at room temperature. After incubation with the secondary antibody, slides were covered with a DAPI nuclear counterstaining solution for 4 min (1:2000 Thermo Fisher Scientific, USA). After the final washing in KPBS, section were cover slipped with Citifluor.

Quantification of pSTAT3

To assess leptin-induced pSTAT3 across the rostraocaudal extent of the VMH, we defined and quantified 4 anatomical levels of VMH and corresponding levels of ARH. The round rostral part of the VMH (level 67, bregma: -1.255 in Allen Mouse Brain Reference Atlas, 2011) was defined as Level 1, and the 3 subsequently caudal VMH sections, separated by steps of 120 μm, were Levels 2 through 4. Sections were imaged at 20x magnification using Zeiss Axioimager Z2 epifluorescence microscope, fitted with Zeiss Axiocam HRm camera, and AxioVision Imaging System (Carl Zeiss MicroImaging GmbH, Germany). We used an exposure time of 270ms together with linear image adjustment for the contrast and brightness. Images were batch processed using ImageJ and a customized macro. For each image, the region of interest (VMH or ARH) was manually selected, and the macro then transformed the image into a binary image by subtracting the background and thresholding the brightness. To separate coalesced cells, the watershed feature of ImageJ was used. Finally, the macro counted pSTAT3-positive cells using a minimum particle size of 30 pixels. To verify the quantification, an image overlay identifying each counted cell was generated and saved.

Measurement of body composition

CT scanning was performed using a La Theta LCT-100. The mice were sutured closed after perfusion, and placed supine in the plexiglass holder with an inner diameters of 48mm. The X-ray source tube voltage was set at 50kV with 1 mA current. After a sagittal image of the whole animal, which was used as an overview about the position of the animal, scans of the entire animal were done. To avoid artifacts, the legs were extended effectively as possible. The region between the vertebrae L1-L6 was evaluated for lean and fat mass. LaTheta LCT-100 software automatically distinguishes between visceral and subcutaneous fat, however each image was examined and corrected if assigned incorrectly.

Statistical analysis

Data are represented as mean ± SEM. Significance was tested using one-way ANOVA or two-way ANOVA, followed by Tukey's multiple comparison, when appropriate. With the exception of the analysis of pSTAT3 quantification, in which all groups were compared, multiple comparisons were only made between mice of the same LepR genotype (i.e. WT/WT vs WT/Tg+ and KO/WT vs. KO/Tg+). A P-value < 0.05 was considered statistically significant. Prism Version 7 for Mac OS X (GraphPad Software Inc., San Diego, CA, USA) was used for analysis.

RESULTS

Leptin sensitivity is restored in the VMH of LepR^{loxTB} x SF1-cre mice

To assess the functional expression of LepR within SF-1 transgenic neurons, we injected fasted mice with leptin to induce pSTAT3. Representative images showing pSTAT3 in the ARH and VMH of all groups are shown in Figure 2A-D. As anticipated, in 8-week old WT/WT and WT/Tg+ mice, we observed leptin-induced pSTAT3 in the ARH (Fig. 2E). Signaling in the VMH was more variable in the WT mice (Fig. 2F), notably in the WT/WT mice, where two mice with ARH-signaling had inexplicably little or no pSTAT3-postive cells in the VMH. In contrast, pSTAT3 immunoreactivity was completely absent in KO/WT mice, as was expected in these LepR-null mice. KO/Tg+ showed a restoration of leptin-induced pSTAT3 in the VMH, but not the ARH. KO/Tg+ pSTAT3 levels were significantly higher than KO/WT in VMH brain levels 2-4. Though we anticipated un-silencing only in a portion of VMH LepR, based on previous studies showing that not all LepR-expressing neurons in VMH co-express SF1, our data show that KO/Tg+ mice have comparable pSTAT3 in the VMH as the WT groups, i.e. there were no significant differences between KO/Tg+ and LepR WT mice at any level of the VMH. To determine if leptin signaling in the VMH diminishes with age, leptin-induced pSTAT3 was again assessed at 20-weeks of age. At this time point, a similar pattern of pSTAT3 expression was observed in the ARH and VMH (Figs. 2G & H).

When the mice were maintained on HFD for 6-weeks, leptin sensitivity was sustained in the ARH and VMH of WT/WT and WT/Tg+ mice, and in the VMH of KO/Tg+ mice (images shown in Figs. 2I-L; quantification shown in Figs. 2M & N). The numbers of pSTAT3-positive cells in the ARH and VMH were similar to chow-fed mice, however a higher dose of leptin was administered (5 mg/kg) to HFD-fed mice, suggesting a blunting of leptin sensitivity.

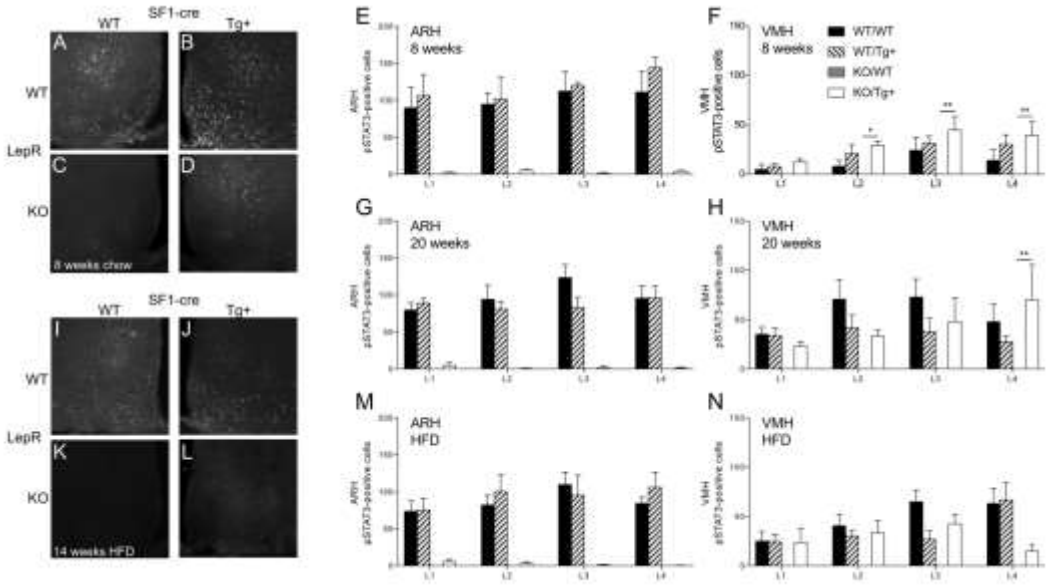


Figure 2: **LepR are only un-silenced in the VMH of KO/Tg+ mice.** Representative images of leptin-induced (3 mg/kg) pSTAT3 immunoreactivity in 8-week old WT/WT (A), WT/Tg+ (B), KO/WT (C), KO/Tg+ (D) mice fed with chow. Mean ± SEM number of pSTAT3 positive cells across four anatomical levels of ARH and VMH in 8-week old (E, F) and 20-week old (G, H) chow-fed mice. Representative images of leptin-induced (5 mg/kg) pSTAT3 immunoreactivity in 14-week old WT/WT (I), WT/Tg+ (J), KO/WT (K), KO/Tg+ (L) mice fed with HFD for 8 weeks. Mean ± SEM number of pSTAT3 positive cells across four anatomical levels of ARH and VMH in 14-week old HFD-fed mice (M, N). Symbols denote significant differences between KO/WT and KO/Tg+; *p<0.05, **p<0.01.

VMH leptin signaling does not ameliorate obesity or increased fat mass

To examine the impact of specific restoration of LepR in the VMH, body weight gain and body composition in chow- and HFD-fed mice were measured. On normal chow diet (Figs. 3A & B), both male and female KO/WT and KO/Tg+ mice were significantly heavier than the WT mice from 6-weeks of age ($p<0.001$ for all comparisons), but were not significantly different from each other. When maintained on HFD for six weeks (Fig. 3C), we observed an early and faster body weight gain, which was comparable, in KO/WT and KO/Tg+ mice. From 6-weeks of age, LepR KO mice were significantly heavier than WT groups ($p<0.001$ for all comparisons), but never different from each other. These effects also did not vary with sex.

To determine whether restoration of LepR in SF1 neurons affected fat deposition, we performed CT scans postmortem in 14-week old mice fed chow or HFD (Figs. 3D & E). Restoration was insufficient to normalize body composition, regardless of maintenance

diet. While lean mass was similar across all groups on both chow and HFD, fat mass was similarly increased in KO/WT and KO/Tg+ mice. The LepR KO groups displayed a notable increase in subcutaneous fat under HFD. The data suggest that increased fat mass underlies increased body weight. Together these results suggest that VMH leptin-signaling does not protect against increased body weight or fat accumulation observed in LepR-null mice, nor does VMH-leptin signaling offer further protection from diet-induced obesity when the mice are placed on HFD.

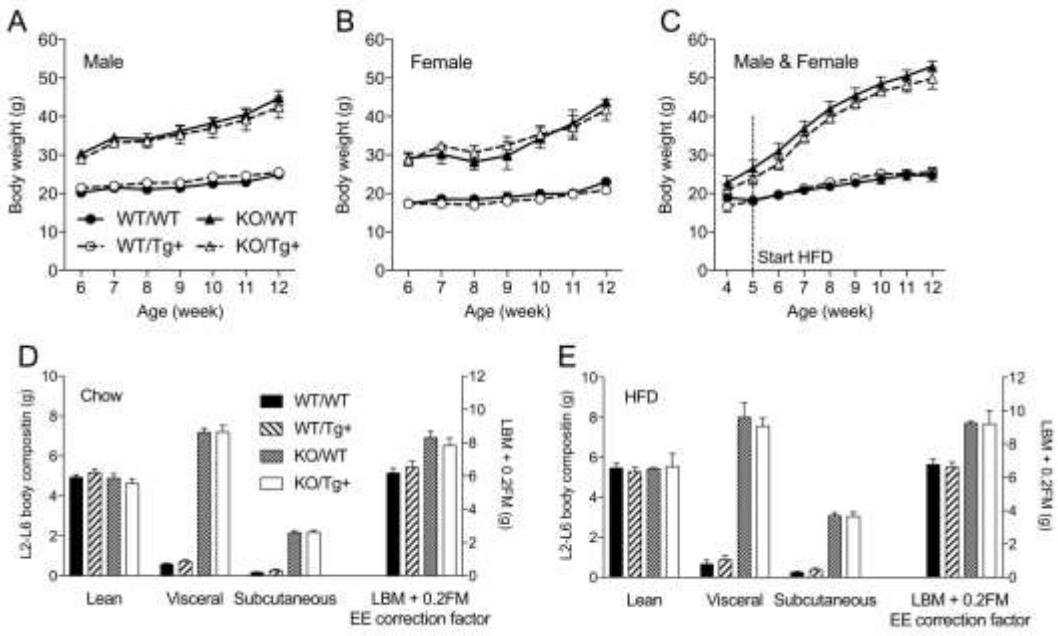


Figure 3: **Un-silencing of LepR in SF1 VMH neurons has no effect on body weight or composition.** Mean ± SEM weekly body weight of male (A), and female (B) mice on chow diet, and male & female mice on high fat diet (C). Mean ± SEM mass of lean, visceral fat, and subcutaneous fat as measured by CT scan in 14-week old mice maintained on chow (D) or HFD (E). D and E also depict the correction factor used to normalize energy expenditure data, which was calculated using lean body mass (LBM) + 0.2 fat mass (FM).

VMH leptin signaling does not normalize energy expenditure nor food intake

We next assessed whether un-silencing of LepR in the VMH protects against reduced energy expenditure and hyperphagia observed in LepR-deficient mice. At the age of 11 weeks, KO/Tg+ and control mice were single housed in metabolic cages and the EE was analyzed. Hourly EE and the average EE for dark and light phases are shown in Figs. 4A & B. In the dark phase, KO/WT and KO/Tg+ mice on chow expended significantly less energy than LepR WT groups ($F[1, 27] = 5.38$, $P < 0.05$), but were not

different from each other. No differences across groups were observed during the light phase (F[1, 27] = 0.03, P = 0.86). We observed a main effect of LepR on RER during both dark and light phases (F[1, 27] = 18.96 and 35.72, P < 0.001 for both
Figs. 4C & D); LepR KO groups exhibited consistently higher RER, but no differences between KO/WT and KO/Tg+ were found. When maintained on HFD, the LepR-deficiency had a more pronounced lowering effect on EE, which was significant during both light and dark phases (F[1, 11] = 17.58 and 5.79, P < 0.01 and 0.05; Figs. 4E & F). However, the presence of VMH LepR had no effect on EE, compared to the KO/WT mice. As anticipated, all groups showed a reduction in RER when maintained on HFD rather than chow (Figs. 4G & H), with no detectable differences across groups in either phase of the light cycle (F[1, 11] = 0.57 and 1.23, P = 0.47 and 0.29). These data suggest that restoration of leptin signaling in the VMH does not improve the diminished EE observed in completely LepR-deficient mice.

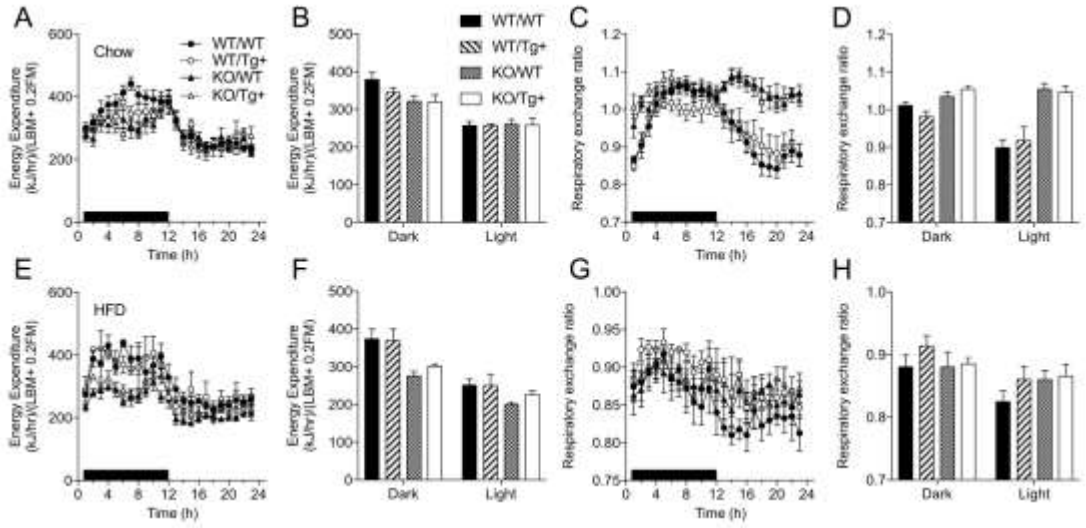


Figure 4: Selective reactivation of LepR in SF1 VMH neurons does not improve energy expenditure (EE) or respiratory exchange ratio (RER). Mean ± SEM energy expenditure (A, B, E, F) and respiratory exchange ratio (C, D, G, H) calculated hourly (A, C, E, G) and during dark and light phase (B, D, F, H) measured in WT/WT, WT/Tg+, KO/WT, and KO/Tg+ mice fed standard chow (A-D) or high fat diet (E-H).

Additionally, 24-h chow intake was similarly increased in KO/Tg+ and KO/WT, compared to LepR WT mice (main effect of LepR, F[1, 26] = 41.43, P < 0.001; Fig. 5A). The increased food intake resulted from an increase in meal size in KO groups (F[1, 26] = 15.60, P < 0.001; Fig. 5B), while there were no differences in meal number across the

four genotypes (F[1, 26] = 0.73, P > 0.05; Fig. 5C). When maintained on HFD, we observed similar differences in food intake between the obese phenotypes and the control mice, but with no differences between KO/WT and KO/Tg+ mice; 24-h intake of HFD was higher in both LepR KO groups (F[1, 24] = 22.66, P < 0.001; Fig. 5D), as a function of increased meal size (F[1, 24] = 18.55, P < 0.001; Fig. 5E), with no change in meal number (F[1, 24] = 3.26, P > 0.05; Fig. 5F). These data demonstrate that VMH leptin signaling is not sufficient to correct the increased intake of chow or HFD or altered meal structure of LepR-deficient mice.

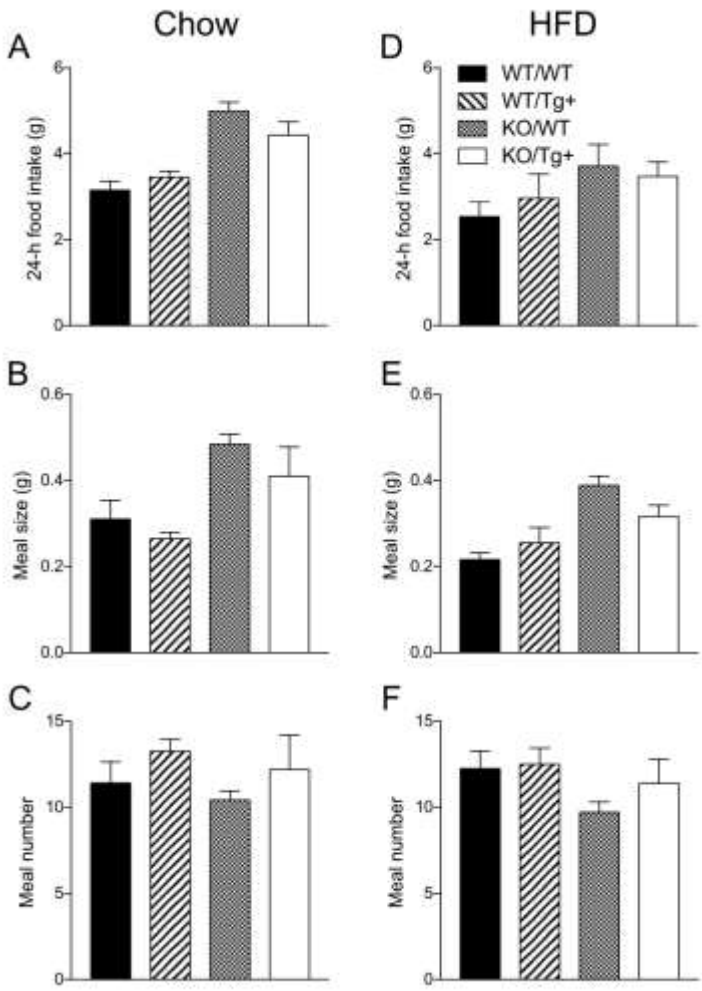


Figure 5: **Selective reactivation of LepR in SF1 VMH neurons does not reduce food intake or alter meal patterns.** Mean ± SEM 24-hour food intake (A, D), meal size (B, E) and meal number (C, F) measured in WT/WT, WT/Tg+, KO/WT, and KO/Tg+ mice fed standard chow (A-C) or high fat diet (D-F).

VMH leptin signaling mildly improves glucose tolerance in HFD-fed mice

To investigate if VMH leptin signaling contributes to glucose handling, we performed igGTT on 8-week and 12-week old mice on chow diet (Figs. 6A & B). There was no significant difference in fasting glucose (0 min time point) across the four groups at either age. At 8 weeks, there was an overall effect of genotype ($F[3, 21] = 4.42$, $P < 0.05$), however no differences between KO/WT and KO/Tg+. At 12 weeks, we observed a stronger overall effect of genotype ($F[3, 37] = 38.76$, $P < 0.001$). At 45 min post-gavage, the KO/Tg+ was significantly higher than the KO/WT group ($P < 0.01$), indicating that VMH leptin signaling actually worsened the ability to clear glucose after the challenge.

To further examine if VMH leptin signaling changes glucose tolerance under conditions of DIO, the igGTT was again performed prior to and after 6-weeks maintenance on HFD (Figs. 6C & D). As with the 8-week chow data, prior to HFD, we again observed a main effect of genotype ($F[3, 24] = 14.71$, $P < 0.001$), but no difference between KO/WT and KO/Tg+ mice. Following 6 weeks on HFD, however, KO/WT showed a delay in blood glucose levels returning to baseline, and were significantly higher than KO/Tg+ mice at 60 min ($P < 0.05$). At the 90 and 120 min time points, only the KO/WT glucose levels were significantly higher than the mice expressing normal LepR. These data indicate, that despite having no effect on obesity or food intake, restoration of LepR SF-1 neurons leads to improved glucose control when mice are maintained on HFD.

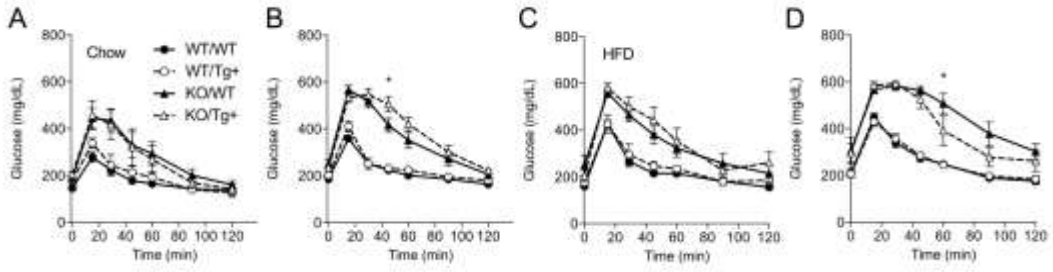


Figure 6: **VMH leptin signaling mildly improves glucose tolerance in HFD-fed mice.** Mean ± SEM blood glucose concentrations measured during intragastric glucose tolerance test (igGTT) in WT/WT, WT/Tg+, KO/WT, and KO/Tg+ mice fed chow diet at 8 weeks (A) and 12 weeks of age (B). igGTT was repeated in a second cohort of mice at 5 weeks of age (C), and age at 12 weeks (D) after 6 weeks on HFD. At dark onset, baseline blood glucose was measured and mice were gavaged with 2 g/kg glucose. Blood glucose was measured at 15, 30, 45, 60, 90 and 120 min post gavage. Symbols denote significant differences between KO/WT and KO/Tg+; *p<0.05.

VMH leptin signaling marginally improves amylin sensitivity

Based on evidence showing that LepR-deficient mice have reduced amylin sensitivity (21) and that the VMH may mediate leptin and amylin interaction in rodents (24), we tested whether VMH leptin signaling would enhance the anorexic response of peripherally administered amylin. The dose-dependent reduction in food intake 1, 2, 4, and 22 h after various doses of amylin is shown in Figs. 7A-D. In order to directly compare the effectiveness of amylin to reduce food intake in KO/WT and KO/Tg+, data from these genotypes were expressed as a percent of baseline food intake (vehicle treatment = 100%; Figs. 7E-H). Two hours after amylin treatment, we observed significant main effects of dose, genotype and an interaction between the factors (F[4, 64] = 3.88 and 3.09, $P < 0.01$ and 0.05 for genotype and interaction respectively; F[1, 64] = 4.90, $P < 0.05$ for genotype). Post-hoc analysis showed a significant difference in the suppressive effects of 100 μg/kg of amylin at 2 h between KO/WT and KO/Tg+ mice ($P < 0.05$; see Fig. 6F). At 1 and 4 h post-injection, there was a significant main effect of amylin dose (F[4, 64] = 7.72 and 6.75 for 1 and 4 h respectively, $P < 0.001$ for both), however no effect of genotype.

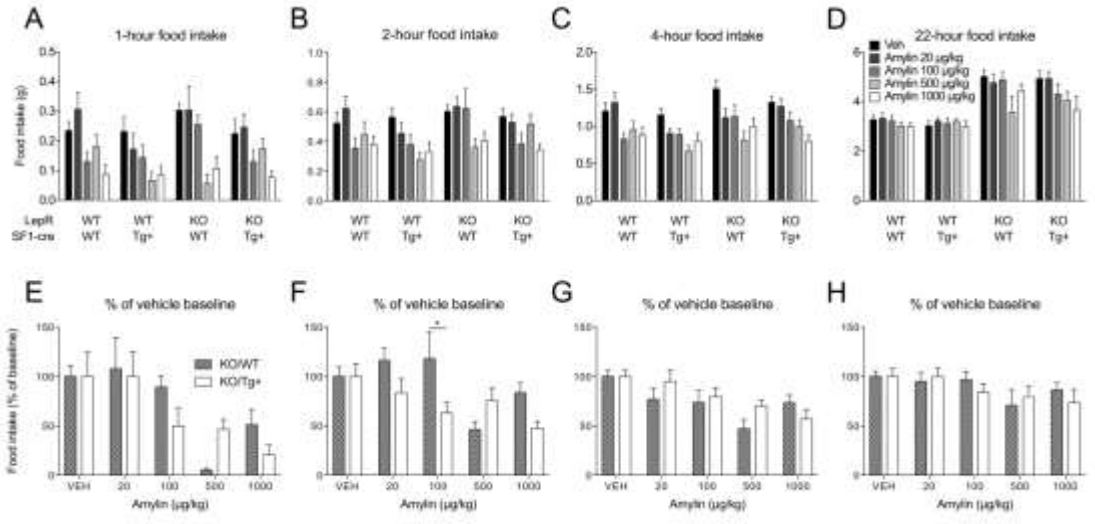


Figure 7: **VMH leptin signaling marginally improves amylin sensitivity.** Mean ± SEM cumulative food intake in WT/WT, WT/Tg and KO/WT, KO/Tg mice at one (A), two (B), four (C) and twenty-two hour (D) after treatment with vehicle, 20, 100, 500, or 1000 μg/kg amylin (IP). Food intake data represented as a percentage of vehicle baseline in KO/WT and KO/Tg+ is shown in E-H. Symbol denotes significant differences between KO/WT and KO/Tg+; *p<0.05.

DISCUSSION

The current study aimed at characterizing the effect of leptin specifically on VMH neurons by un-silencing the innate LepR expressed only in SF1 neurons. While we could demonstrate that leptin-induced pSTAT3 was present only in the VMH and not other hypothalamic nuclei, restoration of LepR in SF1 neurons did not prevent the development of an obese phenotype. VMH leptin signaling did not improve the increased fat accumulation, increased food intake, reduced energy expenditure, nor impaired glucose homeostasis, which was observed in a completely LepR-null KO/WT mouse. Furthermore, while leptin action in the VMH is hypothesized to protect against diet-induced obesity (2), we observed few benefits of VMH LepR signaling when mice were challenged on HFD. As on chow, KO/Tg+ remained as obese as KO/WT mice, again demonstrating increased food intake and reduced energy expenditure. Interestingly, un-silencing VMH LepR appeared to improve glucose tolerance in mice maintained on HFD, suggesting that leptin signaling in SF1 neurons could be relevant for the control of glycemia under pathological conditions, such as obesity.

Our results are surprising provided the numerous reports showing that the SF1 neurons expressing LepR in the VMH are important regulators of energy homeostasis, however exactly how these LepR contribute to this regulation is often inconsistent across these studies. The two initial studies employing genetic deletion of LepR from SF1 neurons provided comparable results (2, 8). Mice deficient in LepR in SF1 neurons were more obese than WT controls, an effect that was more pronounced on HFD, and displayed disturbed glucose tolerance (2). When challenged with HFD, KO mice showed a mild increase in food intake, but a clear impairment in the thermogenic response to the HFD. Together, the data suggest that deficiency in LepR in SF1 neurons leads to increased susceptibility to DIO, which results from impaired control of food intake and energy expenditure (2, 8). The study by Bingham and colleagues also concluded that SF1 LepR were instrumental in the anorexic response triggered by exposure to HFD (2).

While these loss-of-function studies suggest that this population of LepR-expressing neurons is involved in both the regulation of body weight, by altering both food intake and energy expenditure, and the regulation of glucose regulation, experiments employing other methods have produced alternative conclusions. Even prior to the generation of

SF1/LepR-deficient mice, it was shown that the LepR-rich, dorsomedial portion of the VMH sends strong excitatory input to POMC neurons in the ARH, and that these projections are thought to suppress food intake (27). However, while the strength of this excitatory input did appear to wane under fasting conditions, leptin did not have a direct effect. More recently, Meek and colleagues showed that photostimulation of SF1/LepR neurons in the VMH does not alter blood glucose levels (23). Similar stimulation of the entire SF1 population resulted in hyperglycemia, and inhibition of this population prevented recovery from insulin-induced hypoglycemia (23). Based on these results, the authors concluded that SF1/LepR neurons do not regulate the hyperglycemic effect observed when all SF1 neurons were stimulated, and are therefore not mediators of the counterregulatory response following hypoglycemic episodes (23). They also provide the alternative explanation that a subsequent, non-SF1 LepR population inhibits the net output of the photostimulation.

The discrepancies between the loss-of-function studies and these latter reports begin to demonstrate the complexity of the SF1- and LepR-expressing neuronal populations in the VMH. In addition to bearing LepR, SF1 neurons in the VMH also express insulin receptors (InsR). While deletion of LepR from SF1 neurons results in increased sensitivity to HFD and pronounced DIO, deletion of InsR from SF1 neurons protects against DIO when mice are maintained on HFD (16). More recent evidence shows that the LepR and InsR are present in discrete populations within SF1 neurons of the VMH. LepR-expressing SF1 neurons are further divided into populations that are either activated or inhibited by leptin, while insulin application hyperpolarized all InsR neurons (26).

At present, how these discrete populations interact or influence downstream behavioral or physiological output is unclear. Future investigations into the microcircuitry of SF1 neurons within in the VMH and beyond are necessary to understand why reactivation of native LepR on SF1 neurons in the VMH has little consequence on the obese phenotype of LepR-null mice. For instance, we now know that the VMH contains SF1 neurons that are both depolarizes and hyperpolarized by leptin (26), but it was also shown that up to 20% of leptin-activated neurons in the VMH are negative for SF1 (8). Based on these data, we could speculate that in our model, where only LepR in SF1-postive neurons are reactivated, perhaps the opposing responses of these two populations to leptin results in

neutralizing either subpopulation's net effect on energy homeostasis. Furthermore, while it is reported that leptin-depolarized and –hyperpolarized SF1 neurons occur in relatively equal distribution, the contribution of non-SF1 neurons expressing LepR should not be disregarded. It is possible that activation of this additional LepR population in the VMH could be critical in determining the final behavioral and physiological output. This hypothesis aligns with the alternative conclusion put forth by Meek and colleagues (23), mentioned above.

Another aspect to consider is whether our model of LepR-reactivation in SF1 neurons preserves the genomic machinery that underlies the proposed protective effect from HFD-induced obesity (26). We demonstrated that leptin induced pSTAT3 in the VMH of KO/Tg+, to levels comparable to both WT control groups, indicating that we restored the LepR signaling required for leptin to phosphorylate STAT3. This readout of acute LepR function, while commonly used, doesn't provide information about the downstream signaling which is necessary to drive chronic changes at the transcription level, such as pathways involving the transcription factor, FOXO1. Positioned downstream of the PI3K/pAkt signaling pathway, FOXO1 is characterized as a negative regulator of leptin action. Under conditions of increased leptin activity, elevated levels of pAkt lead to increased phosphorylation of FOXO1, preventing its translocation into the nucleus, which in turn allows leptin's effect on energy balance. Deletion of FOXO1 from VMH SF1 neurons produced a mouse exhibiting increased leptin sensitivity, with increased energy expenditure, improved glucose metabolism and resistance to HFD-induced obesity (14). The absence of FOXO1 in the VMH also produced elevated mRNA and protein levels of SF1 within the VMH, and SF1 was shown as a necessary promoter of leptin's beneficial actions (14). Based on these findings, we could hypothesize that despite the restored leptin-induced pSTAT3 observed in our KO/Tg+, the mice exhibit increased FOXO1, which prevents SF1 transcription and restricts leptin from chronically improving the metabolic state of the otherwise LepR-null mice. Additional studies exploring if the PI3K/pAkt/FOXO1 signaling pathway is modified in our model are required to verify this.

In contrast to the predominately null phenotype, KO/Tg+ mice showed a slight improvement in glucose tolerance compared to the KO/WT mice, but only when they were maintained on HFD. Because the KO/Tg+ and KO/WT mice on HFD did not

differ in body weight or food intake, it is possible that restoration of LepR in the VMH directly influenced insulin signaling in the VMH to alter glucose metabolism. It was previously shown that LepR-deficient db/db mice have reduced insulin-induced pAkt in ARH neurons, corroborating that functional leptin signaling is important for insulin signal transduction (17). Interestingly, activity of insulin-responsive SF1 neurons was shown to also vary with diet. These neurons are overactive specifically under conditions of HFD-feeding and concomitant hyperinsulinemia, which results in elevated PI3K activation and a proposed inhibition of the glutamatergic VMH projections to ARH POMC neurons (16). While VMH insulin signaling should not theoretically be disrupted in any of our experimental groups, it is possible that the effect on glucose tolerance we observed in the KO/Tg+ mice results from an interaction between the HFD-induced hyperactivity in SF1 InsR neurons and enhanced pAkt signaling in these neurons, which could be a result of unsilencing of VMH LepR.

Our results also differ to what was observed after un-silencing native LepR in POMC neurons in the ARH. In this report, which initially validated the LepR^loxTB mouse model, restoration of LepR signaling in ARH POMC neurons improved the overall metabolic status (1). The most pronounced effect of reactivating LepR in POMC neurons was the complete correction of the severe hyperglycemia observed in the LepR-null control. This effect was primarily attributed to a normalization of glucagon levels, though POMC LepR un-silencing also resulted in improved insulin action and lower basal insulin levels (1). Though we did not assess blood glucose or metabolic hormones over the same time course as Berglund and colleagues, the blood glucose levels measured prior to glucose administration during the igGTT were not different across our four experimental genotypes. Restored POMC LepR signaling also resulted in a modest correction in obesity and fat mass, but this effect was only significantly different from LepR-deficient mice after 13 weeks of age (1). Our model failed to show any correction in body weight or fat mass, which we measured until 14 weeks of age. While it is possible that a difference would have emerged later in life, body weight data collected from the cohort of mice sacrificed at 20 weeks of age to assess leptin-induced pSTAT3, also show no difference between KO/WT or KO/Tg+ mice (61.4 ±3.0 g vs. 63.3 ± 3.8 g, respectively).

Discrepancies between the Berglund study and ours suggest that the POMC LepR-expressing population in the ARH plays a more critical role in mediating leptin's effect of energy balance than SF1 LepR neurons in the VMH. It is also possible that leptin signaling via the SF1 neurons requires additional populations of functional LepR, such as ARH LepR, in order to induce changes in metabolism. It is established that postnatal ARH leptin signaling is required for correct organization of hypothalamic circuitry (4). LepR-deficient db/db mice show disturbed neuronal projections from the ARH to the paraventricular nucleus of the hypothalamus, which is a critical pathway in the control of energy balance (3). This phenotype is presumably replicated in our KO/WT and KO/Tg+ mice, which lack ARH LepR. As the ARH is a known target of VMH projections (27), and a potentially critical mediator of the VMH's effects on energy balance, it is possible that the disturbed ARH circuitry halts downstream transmission of the leptin signal generated in the VMH of KO/Tg+ mice. A more direct relationship between ARH and VMH LepR signaling might also exist; co-activation of these ARH POMC neurons by leptin could be necessary to transduce the hypothesized anorexic action that follows SF1 leptin signaling. Interestingly, reactivation of LepR exclusively in POMC neurons also did not correct the hyperphagia observed in LepR-null mice (1), which would support the hypothesis that both LepR populations are required for leptin's effect on food intake. Certainly, the fact that reactivation of neither LepR population completely corrects metabolic dysfunction supports the idea that leptin action does not depend on a single receptor population, but rather a distributed network of leptin-responsive nuclei, likely involving both the hypothalamus and the hindbrain (11).

In fact, the influence of hindbrain leptin signaling could be quite important for determining meal size and the salience of various satiating hormones (12, 25). We showed here that VMH LepR signaling had only a minor effect on the food intake-suppressive effect of the pancreatic hormone amylin. These results are in contrast to previous findings suggesting that the VMH is a critical locus for the interaction of amylin and leptin to potentiate either hormones ability to reduce body weight and food intake (24, 30). Of course the aforementioned explanations why VMH LepR-signaling did not improve overall metabolic dysfunction could also underlie the negligible effect of VMH LepR signaling on amylin sensitivity. Or it could simply be that hindbrain, rather than hypothalamic, LepR are responsible for modulating amylin signaling. Our previous work showed that LepR-deficient mice (db/db) and rats (ZDF) have reduced amylin-induced

satiation, and that db/db mice also exhibit reduced amylin-induced Fos in the primary target nucleus of amylin, the area postrema (AP; 21). We also showed that single neurons within the AP express the RNA for all amylin receptor components and the LepR (20), demonstrating the potential of this hindbrain LepR population to influence amylin signaling.

In summary, we found that LepR signaling specifically in SF1 neurons of the VMH is not sufficient to mediate the metabolic effects of leptin. VMH LepR signaling, however, improved glucose homeostasis under HFD conditions. Our findings suggest that SF1 neurons in the VMH might contribute to the regulation of leptin-mediated glucose homeostasis following diet-induced obesity. Further studies should address whether leptin signaling in the VMH plays a subordinate role in metabolic regulation or if its functions depend on connection within other leptin-regulated nuclei in the brain.

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